Drought has a major negative impact on agriculture productivity and food supply. Plants loose water by a process called transpiration through epidermal pores called stomates. The opening and closing of stomates is mediated by changes in the shape the two guard cells that surround each stomate. While the hormone abscisic acid (ABA) has long been known to participate in protective strategies by regulating transpiration and growth, drought also affects the levels of another hormone gibberellin (GA). Previous studies, including our studies with tomato, have shown that reducing GA responses through manipulation of hormone levels or signaling pathway components promotes tolerance to drought. GA acts by triggering the destruction of DELLA proteins, which repress most if not all GA responses. Tomato has one DELLA protein called PROCERA (PRO) and results obtained prior to initiating this project suggested that PRO promoted drought tolerance, at least in part, by a mechanism that likely involves altering guard cell activity and sensitivity to the hormone ABA but it was not known if PRO modulated transpiration by acting in the guard cell or throughout the leaf. Since DELLA proteins affect growth one hypothesis was that DELLA affected transpiration through its effects on leaf size. The primary objective of this project was to test the hypothesis that increasing PRO abundance increases drought tolerance by mechanisms that include promoting ABA responses and expression of stress-related genes and that this effect is independent of its effect on growth. The data from this project shows that PRO acts in guard cells to promote stomatal closure and reduce water loss in response to water deficiency by increasing ABA sensitivity. A loss-of-function *pro* mutant exhibited increased stomatal conductance and rapid wilting under water deficit stress. In contrast, transgenic tomato overexpressing constitutively active stable DELLA proteins (S-della) displayed the opposite phenotype. The effects of S-della on stomatal aperture and water loss were strongly suppressed in the ABA-deficient mutant *sitiens*, indicating that these effects of S-della are ABA dependent. While DELLA had no effect on ABA levels, guard cell ABA responsiveness was increased in S-della and reduced in *pro* plants. Expressing S-della under the control of a guard-cell-specific promoter was sufficient to increase stomatal sensitivity to ABA and to reduce water loss under water deficit stress but had no effect on leaf size or size of any other organ. This result indicates that increasing DELLA activity promotes stomatal closure independently of its effect on growth. To identify DELLA-regulated genes that may be modulate guard cell activity, we performed two transcriptomic studies. One study examined the effects of PRO on the ABA regulated gene expression in shoots of wild type, *pro* and S-della plants and the second examined the gene expression in guard cells from plants these genotypes. These studies identified a number of DELLA-regulated genes some of which are also drought- and/or ABA-regulated. Preliminary studies suggest that overexpression of one of the PRO up-regulated genes reduced water loss. Since mutants with increased PRO activity did not exist in tomato, another goal of the project was to gene edit PRO to create mutants that expressed a stabilized version of PRO. Two dwarfing PRO alleles with mutations predicted to increase PRO stability were generated using the CRISPR/Cas9 system and characterized. The mutants are extreme dwarfs and do not respond to exogenous GA.

We coordinated the project by frequent exchanges of emails that exchange and discuss scientific results and review the goals and objectives of the project. The labs freely shared results, methods and reagents. In June 2016, the US PI (Olszewski) met with Ido Nir, a graduate student from the Weiss lab, at the International Conference on Plant Growth Substances that was held in Toronto and reviewed new results from both labs. In May 2017, David Weiss visited Olszewski lab to discuss the results and to write the first manuscript. At the end of this year, we (D.W. and N.O) published this paper in The Plant Cell. Recently we publish our second paper together on the generation of *pro* gain-of-function CRISPR-Cas9-derived mutant in Plant Biotechnology Journal.

Drought has a major negative impact on agriculture productivity and food supply. Plants loose water by the process of transpiration through epidermal pores called stomates. The opening and closing of stomates is mediated by changes in the shape of the two guard cells that surround each stomate. While the hormone abscisic acid (ABA) has long been known to participate in protective strategies by regulating transpiration and growth, drought also affects levels of another hormone gibberellin (GA). Previous studies, including our studies with tomato, have shown that reducing GA responses through manipulation of hormone levels or signaling pathway components promotes tolerance to drought.

GA acts by triggering the destruction of DELLA proteins, which repress most if not all GA responses. Tomato has one DELLA protein called PROCERA (PRO) and results obtained prior to initiating this project suggested that increasing PRO activity increases drought tolerance. Therefore manipulation of GA signaling is a potential avenue for improving crop drought tolerance. However more must be learned about the role of GA signaling and DELLA proteins in drought tolerance for this potential to be achieved.

The primary objective of this project was to test the hypothesis that increasing PRO abundance increases drought tolerance by mechanisms that include promoting ABA responses and expression of stress-related genes and that this effect is independent of regulation of growth. Another goal was to learn if PRO affected transpiration by acting in the guard cells alone or by acting in other cells. Since DELLA proteins affect growth, one hypothesis for how PRO affects growth was that DELLA affected transpiration through its effects on leaf size. Finally, we explored the potential of gene editing to mutate and increase the activity of PRO.

To examine PRO's effect on plant water status, we tested the response of the DELLA loss-of-function tomato mutant *pro* to water deficit stress. When water was withheld *pro* plants wilted sooner than wild-type. Microscopic analysis of leaves of well-watered plants revealed that stomatal opening was larger in *pro* versus M82 plants, which resulted in the mutant having a higher stomatal conductance and transpiration rate. These results support the hypothesis that PRO reduces stomatal aperture.

Since mutants with a stabilized (overactive PRO) were not available when the project was initiated, transgenic tomato plants overexpressing a stable version of an Arabidopsis DELLA protein RGA (S-RGA) or stabilized PRO (S-PRO) were created and characterized to determine the effect of increasing DELLA activity on stomatal function. Expression of S-RGA and S-PRO both caused dwarfism but the dwarfism was less severe for S-RGA plants. When water was withheld, S-RGA plants wilted more slowly than non-transgenic plants. Additionally, stomata of the transgenic plants closed when the soil water content was higher than at which stomates of non-transgenic plants closed. Well-watered S-RGA plants had significantly a lower transpiration rate. Similar results were obtained with transgenic plants expressing S-PRO. These results indicate that increasing DELLA activity has the potential to improve drought tolerance but increasing the activity throughout the plant reduces the stature of the plant, which depending upon the degree of dwarfism can be undesirable.

To determine the contribution of leaf size to water loss, transgenic plants expressing S-PRO early in leaf development but not later in leaf development were created. Leaves of these plants were much smaller but stomatal properties and response to withholding water were similar to non-transgenic controls plants. We next determined if expression of S-PRO in guard cells alone was sufficient to improve response to decreased water availability. Transgenic plants expressing S-PRO only in guard cells were created. The stature of transgenic plants was identical to control plants. However, under water-deficit conditions, control plants wilted before the transgenic plants. These results clearly demonstrate that it is possible to manipulate DELLA activity to improve the plant's response to water deficiency without affecting plant stature.

S-PRO was introgressed into the ABA-deficient mutant *sitiens* (*sit*) to test the hypothesis that ABA signaling is required for S-PRO action. Upon drought treatment, *sit* and S-PRO *sit* plants began to wilt sooner than wild-type, indicating that ABA signaling is required for S-PRO action. To determine if DELLA affects ABA signaling, several responses to ABA treatment were examined in wild-type, *pro* and S-RGA. Relative to wild type, ABA-induced stomatal closure, reduction in transpiration and induction of

gene expression was reduced in *pro* and enhanced in S-RGA plants. These results show that DELLA has a role in ABA responses.

To identify DELLA-regulated genes that may be modulate ABA responsiveness and guard cell activity, we performed two transcriptomic studies. One study examined the effects of PRO on the ABA-regulated gene expression in shoots of wild type, *pro* and S-RGA plants. In this experiment, the different genotypes were treated with ABA or a control solutions and changes in gene expression two hours after this treatment were assessed. The second experiment examined the gene expression in guard cells from these plants. Together these studies identified a number of DELLA-regulated genes some of which are also drought- and/or ABA-regulated. Preliminary studies suggest that overexpression of one of the PRO-up-regulated genes reduced water loss.

When this project was initiated there were *pro* loss-of-function mutant but there were no mutants with increased PRO activity. Gene editing using CRISPR/cas9 was used to create two new *PRO* alleles that encode a mutant protein that is not degraded by GA signaling. Both alleles cause extreme dwarfism and increased seed dormancy and one allele (PRO^{GF8}) was characterized further. In contrast to wild-type, PRO^{GF8} did not respond with increased growth when sprayed with GA suggesting it was completely unresponsive to GA. However the height of PRO^{GF8} was reduced to a very small extent when they were treated with a GA biosynthesis inhibitor and this effect was reversed by treatment with GA. Based on these results, we believe that PRO^{GF8} is nearly insensitive to GA. While PRO^{GF8} will be a valuable research tool for understanding PRO function in tomato the reduction in growth and seed germination are too extreme for it to be useful in crop plants. Therefore, we have created transgenic plants where the PRO^{GF8} promoter is being mutated to reduce it expression level to produce mutants with less extreme dwarfism.

There were two changes to the Work Plan.

1) ChIP experiments to identify direct targets of PRO were to be performed by the Olszewski lab as part of Objective 2. This involved developing a new method for precipitating PRO bound chromatin that involved expressing PRO tagged with a promiscuous biotin ligase. In this strategy, the promiscuous biotin ligase would biotinylate PRO-associated chromatin proteins which would be precipitated using streptavidin. Pilot experiments performed in Arabidopsis indicated the activity of the promiscuous biotin ligase was insufficient for this strategy to work.

2) Objective 4 was to determine the effect of drought on PRO accumulation. This was not performed. Instead the transcriptomic analysis with isolated guard cells that are described in the achievements were performed.